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A DIFFERENCE IN NITROSYL-HEMOGLOBIN FORMATION AMONG ANIMAL SPECIES

AN INTERSPECIFIC DIFFERENCE IN NITROSYL-HEMOGLOBIN FORMATION IN ANIMALS

[Issakna chisso hemogurobin seisei shuzokusa]

by

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Nitrosyl-hemoglobin (NOHb) was detected by electron spin resonance spectrometry from the blood of mice and rats exposed to 7 ppm nitric oxide, but in rabbits it was detected only when sodium dithionite was added to the blood. To clarify this interspecific difference, some comparative experiments were conducted using the blood of mouse and rabbit. Rabbit blood showed higher affinity to oxygen and smaller production of NOHb after addition of nitrite compared to the mouse blood. These results may explain the reason NOHb was not detectable in rabbit blood during the exposure to nitric oxide.

Introduction

It is by now well known that nitrogen monoxide (NO) combines with hemoglobin (Hb) *in vitro* to form NOHb which may then be measured by electron spin resonance spectrometry (ESR) [1]. In our latest experiment, we attempted to determine quantitatively the nitrosyl-hemoglobin (NOHb) formed in the blood of animals exposed to NO gas. The results indicated an interspecific difference in the formation of NOHb between mice and rabbits. We proceeded

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with a comparative examination of mice and rabbits upon the belief that the above finding was significant for proper selection of experimental animals and proper evaluation of the toxicity in future animal experiments with NO, and that the NOHb formation mechanism may be deduced through the clarification of the causes of such an interspecific difference.

Regarding the acute, high-concentration effects of nitrogen oxides, Pflesser reported (1935) that while mice exposed to NO exhibited such symptoms of the central nervous system as spasms and paralysis and the formation of methemoglobin, those exposed to NO_2 were found to have pulmonary edema [2]. This finding may conceivably be attributed to a physical difference in which NO, which is less likely to be trapped in the respiratory apparatus, would invade the body system while NO_2, being more water-soluble than NO, would mainly affect the respiratory apparatus. Comparing the acute toxicities of NO and NO_2, Pflesser concluded that NO was four times as toxic as NO_2 on the basis of his finding that the minimum lethal concentration of NO was 310 ppm while that of NO_2 was approximately four times that amount. In contrast, Gray (1959) obtained data indicating that the LC_{50} level in rats exposed to NO_2 for four hours was 88 ppm and, comparing this with Pflesser's data that 300 ppm of NO was non-lethal, he concluded that NO_2 was several times more lethal [3]. However, in Pflesser's NO experiment, which was compared with Gray's experiment with NO_2 for the basis of this conclusion, not only the experimental conditions but also the experimental animals were different. Any comparison of toxicity requires that the species of the experimental animals being compared are the same so that this conclusion by Gray is rather questionable. We felt it necessary to point out this questionable

aspect of Gray's report at this time since the report which has been cited widely in recent years has been one of the reasons behind assessments that NO is less toxic than NO_2.

Detection of NOHb

The exposure to NO and the detection of NOHb were carried out by the following procedures. When pure NO gas contained in a cylinder is diluted with air, NO_2 is formed by oxidation. By filtering this NO_2 through soda lime prior to the introduction of the gas into the exposure chamber, we succeeded in removing most of this NO_2. When the NO level in the chamber was 6.6 ppm, that of NO_2 was 0.5 ppm (Saltzman's method). After one hour of exposure to these concentrations in the chamber, the experimental mice (JCL-ICR, 2-month-old, ♀) were taken out of the chamber and blood samples were collected immediately for their orbital venous plexus. The blood samples were sealed inside quartz tubes 3 mm in inner diameter together with nitrogen, and were frozen right away with a dry ice-acetone solution, after which the NOHb levels were measured by ESR (temperature - 100°C, modulation 100 kHz, modulation amplitude 5 gauss). Fig. 1 shows an ESR spectrum of NOHb from one of the exposed mice. This spectrum coincided with the spectrum of NOHb prepared in vitro from mouse blood. In the figure, DPPH indicates the standard substance with g = 2.0036. In contrast to the case of the mice, the spectrum could not be detected in the untreated blood samples collected from the auricular vein of rabbits (2.0 - 2.5 kg, ♂) which had been exposed in the same manner. We were able to obtain the NOHb spectra (Fig. 2) through reduction by adding $Na_2S_2O_4$ to the samples. The detection of NOHb by ESR in the blood of animals exposed to NO has already been studied by

Sancier (1962) who reported that NOHb could not be detected in the blood of rats exposed to ten ppm of NO for nine days and ~~100 ppm~~ for one day ~~respectively~~ [4]. We attributed this disagreement with our results to differences in the blood sampling conditions and the measurement conditions as well as to the possible involvement of interspecific differences between animals.[1]

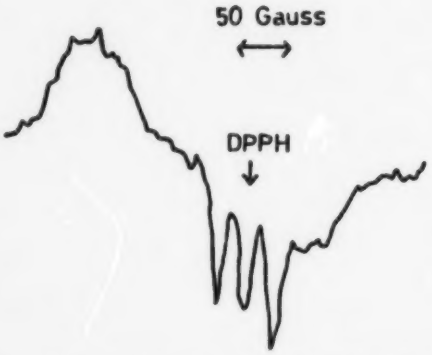

Fig. 1. ESR spectrum of NOHb of mouse exposed to NO

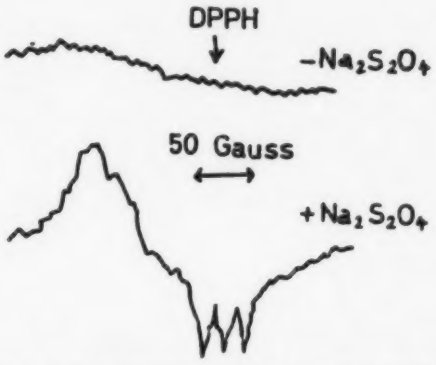

Fig. 2. ESR spectrum of NOHb of rabbit exposed to NO

1. In a follow-up experiment conducted later, about the same amount of NOHb was detected in the blood of rats, which were exposed to NO of the same concentration as in the cases of the rats and mice, as in the blood of mice. This finding led us to the conclusion that the disagreement with the result of Sancier et al., was attributable to the blood sampling conditions and the measurement conditions but not to an interspecific difference.

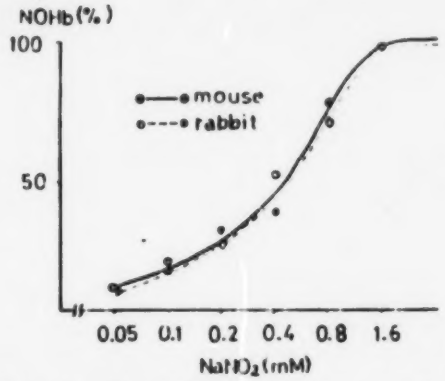

Fig. 4 Formation of NOHb by addition of $NaNO_2$ and excess $Na_2S_2O_4$ to 0.1 *mM* Hb solution

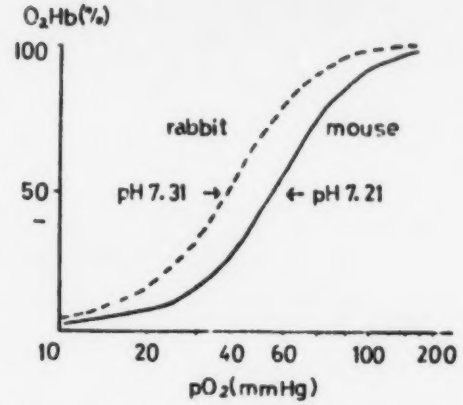

Fig. 4. Oxygen dissociation curve of whole blood

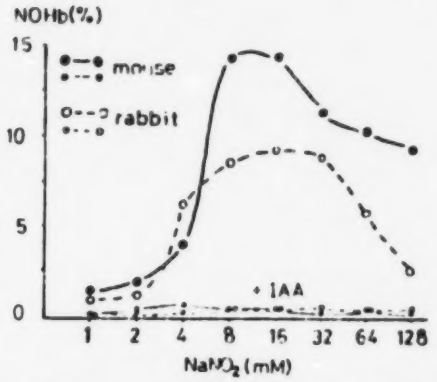

Fig. 5. Formation of NOHb by addition of $NaNO_2$ to red cell suspension (Hb 2 *mM*)

Principal Factors behind Interspecific Difference

In an effort to determine the principal factors causing the interspecific difference in the formation of NOHb between mice and rats, we examined the following three aspects.

1. Comparison in terms of the affinity of Hb to NO.

Should there be a difference between the two species in the terms of the affinity of their Hb to NO, the amounts of NOHb formed would obviously have to be different. Since it would have been technically difficult to add

NO gas in detectable quantities to Hb, we adopted the method of adding $NaNO_2$ in steps to the sample Hb solutions in the presence of excess $Na_2S_2O_4$ and then comparing the amounts of NOHb thus formed. This method is based on the assumption that the amount of NO in the Hb solutions would be dependent on the amount of $NaNO_2$. Since there was no significant difference between mice and rabbits in the amounts of NOHb formed as indicated by Fig. 3, we concluded that there was no difference in the affinity of Hb to NO between the two species. We nevertheless intend to pursue the matter further using an NO gas system.

2. Comparison in terms of the oxygen affinity of Hb

In such a system as the blood circulation in which oxygen is present, the oxygen plays an antagonistic role with respect to the bonding of NO with Hb so that one may assume that the greater the bonding strength of Hb with oxygen, which is to say its oxygen affinity, the smaller would be the amount of NO combining with the Hb. We therefore sought the oxygen dissociation curves with respect to whole blood samples of normal mice and rabbits by means of a DCA-1 radiometer and found, as shown in Fig. 4, that the P_{50} point was at 39 mmHg in the case of rabbits while it was at 56 mmHg in the case of the mice. In addition, the actually measured values for the oxygen saturation level of Hb came out to 40% for the blood sampled from the orbital venous plexus of the mice and 80% for the blood sampled from the auricular veins of the rabbits. In view of this finding that both the oxygen affinity of Hb and the Hb oxygen saturation level of blood samples were higher in rabbits than in the mice, it is believed that there may be inhibition of NOHb formation in the case of the rabbits. This deduction

conforms to the fact that the deoxygenation of the blood of exposed rabbits with $Na_2S_2O_4$ resulted in the formation of NOHb.

3. Comparison in terms of the amount of NOHb formed with the addition of nitrite.

In addition to the pathway of direct reaction between the NO absorbed and the Hb, one may assume the existence of the following formation pathway on the basis of the fact that the addition of nitrite to the whole blood also results in the formation of NOHb [5, 6].

$$NO \xrightarrow{O_2} NO_2 \xrightarrow{H_2O} NO_2^- \xrightarrow{Hb} NO_2^-\text{-MetHb} \xrightarrow{\text{reducer}} NOHb$$

In order to examine the difference in the amounts of NOHb formed by this pathway, we added $NaNO_2$ in steps to red cell sample suspensions and measured the amounts of NOHb formed. As shown in Fig. 5, the maximum value of the amount of NOHb formed for a mouse was 1.6 times that for a rabbit. The participation of an endocellular enzymic reaction in this reaction was suggested [5] by the fact that such a reaction does not occure with the Hb sample solution as well as by the fact that the reaction can be almost completely prevented by the addition of 0.1 M monoiodo-acetic acid (IAA) to the red cell suspension. We also observed that the amount of NOHb diminished when the amount of nitrite reached more than eight times that of the Hb, and this is believed to be attributable to the inhibition caused by the bonding of the nitrite with the enzyme protein in the Hb.

From the above results, it was deduced that the principal factors behind the interspecific difference observed in the formation of NOHb between the mice and the rabbits were the difference in the oxygen affinity of Hb and

the difference in the endocellular reductase activity. It would seem essential to take these factors into account during NO toxicity experiments with respect to the selection of the experimental animals and the evaluation of the toxicity levels in them.

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